

Q¹ ~~FIGURE 54~~ Sequence similarity between SSeCKS and the Abl-binding domain in pRb. Identical a.a. residues (vertical lines) or similarly charged residues (colons) are shown for the SSeCKS and newt Rb (Genbank accession # Y09226) (SEQ ID NO: 21) proteins.

Please **amend** the paragraph on p.40, lines 1-11 with the following paragraph:

Q² In a specific embodiment of the invention, SSeCKS fusion polypeptides may be designed that are capable of anchoring cyclin D to the cytoplasmic region of the cell thereby preventing translocation of cyclin D into the nucleus and induction of cell proliferation. Such fusion polypeptides comprise a SSeCKS cyclin D binding domain and a protein domain capable of anchoring the fusion protein within the cytoplasm. As indicated herein, the SSeCKS cyclin binding domain maps to a region designated CY. The SSeCKS gene encodes two closely spaced CY domains, KKLFSXXXXKKLSG (SEQ ID NO:22) [(K/R)(K/R) followed by two non-polar residues. Anchoring domains include those domains that target binding to, for example, structural membrane proteins, cytoskeletal components or cellular organelles located within the cytoplasm (See, Lester et al., 1997, Recent. Prog. Horm. Res. 52:409-29; Diviani et al., 2001, J. Cell. Sci. 114:1431-7).

Please **amend** Table 1 on p. 60 as follows:

Table 1. Proposed PKC phosphorylation sites in SSeCKS: Comparison with known calmodulin-binding and PKC phosphorylation sites in the MARCKS protein family and in myosin light chain kinase.

	<u>Sequence</u>	<u>CaM binding</u>	<u>Actin binding</u>	<u>PKC binding</u>
MARCKS (bovine/chicken)	¹⁵³ KRFSSKKSFKLSGFSFKKNKKEA ₁₇₇ (SEQ ID NO:11)	+	+	+
MARCKS (mouse)	KRFSSKKSFKLSGFSFKKSKKEA (SEQ ID NO:12)	+	+	+
MacMARCKS/F52	KKFSSKKPFKLSGFSFR (SEQ ID NO:13)	+	+	+
Myosin light chain kinase	KRRWKKAFAIVSAAARFKKC (SEQ ID NO:14) WAGWRKK (SEQ ID NO:20)	+	-	?
SSeCKS-1 (rat)	²⁷⁹ ETTSSFKKFFTHGTSFKKSKEDD ₃₀₇ (SEQ ID NO:15)	?	?	+
SSeCKS-2 (rat)	⁵⁰⁴ KLFSSSGLKKLSGKKQKGKRGGG ₅₂₆ (SEQ ID NO:16)	?	?	+
SSeCKS-3 (rat)	⁵⁹² EGITPWASFKKMVTTPKKRVRPS ₆₂₄ (SEQ ID NO:17)	?	?	+
SSeCKS-4 (rat)	⁷⁴¹ EGVSTWESFKRLVTPRKKS SKL ₇₆₆ (SEQ ID NO:18)	?	?	+
3/4 - Consensus	EGV W SFKK VTPKKK K I R R R R (SEQ ID NO:19)			

Please **amend** the paragraph on p.65, lines 11-17 with the following paragraph:

The ability of SSeCKS to associate with plasma membrane sites is predicted by an N-terminal myristylation signal, MGAGSSTEQR (SEQ ID NO:23), which is similar to signals encoded by retroviral GAGs and the HIV nef product (Anderson and Pastan, 1975, Adv.

Q4 Cyclic Nucl. Prot. Phosph. Res. 5:681). This sequence lacks the Cys-3 residue shared by members of the src and G α family which are also palmitylated, with the exception of G α^t /transducin, the signal of which is quite similar to that of SSeCKS and which is myristylated only. Indeed, SSeCKS was demonstrated to be myristylated by in vivo labeling (Figure 28).

Please **amend** the paragraph on p.102, line 18 to p.10³~~2~~, line 17 with the following paragraph:

Q5 SSeCKS2 fragments (a.a. residues 389-552) were generated by PCR amplification and cloned in pBluescript II KS (Stratagene)(40). Mutations in two potential CY motifs were generated using a TransformerTM Site-Directed Mutagenesis Kit (Clontech). A unique restriction site in pBluescript, Sca I, was chosen as a selection marker (*Sca* I to *Stu* I). "Trans" and "switch" selection primers were: 5'GTGACTGGTGAGGCCTCAACCAAGTC (*Sca* I to *Stu* I) (SEQ ID NO: 24) and 5'GTGACT GGTGAGTACTCAACCAAGTC (*Stu* I to *Sca* I) (SEQ ID NO: 25), respectively. Trans-mutagenic primers were as follows:

5'GGAAGTCCCTTGTCGAGCCTCTTCAGTAGC (first KK to SS) (SEQ ID NO: 26),

5'GCTC AGGCTTAAGCTCGCTGTCTGGG (second KK to SS) (SEQ ID NO: 27),

5'CCCTTGAAGAAAAGC TTCAGTAGC (first L to S) (SEQ ID NO: 28),

5'GGCTTAAAGAAGTCGTCTGGGAAG (second L to S) (SEQ ID NO: 29). Switch-mutagenic primers were: 5'CCCTTGTCGAGCAGCTTCAGTAGC (first L to S) (SEQ ID NO: 30) and

5'GGCTTAAAGCTCGTCGTCTGGGAAG (second L to S) (SEQ ID NO: 31). After

denaturation, the target SSeCKS2 plasmid was annealed with primers, followed by synthesis of the mutant strand DNA. Primary selection was carried out by restriction digestion. The mutated plasmid was amplified, and then was subjected to a second round of restriction enzyme

Q5 digestion. All mutations were confirmed by sequencing using Sequenase 2.0 kits (US Biochemicals). The resulting SSeCKS2 variants were spliced back to pGEX 5x-1 for fusion protein expression. BL21 (DE3) pLysS bacteria (Novagen) were transformed with these constructs, grown in LB/Amp medium containing 20 mM glucose at 37°C, and GST-fusion protein induced and purified as described previously (Lin et al., 1996, J. Biol. Chem. 271:28430-28438; Sambrook et al., 1989, Molecular cloning: a laboratory Manual, Cold Spring Harbor Laboratory Press).

Please **amend** the paragraph on p.104, line 14 to p.105, line 3 with the following paragraph:

Q6 Peptides were synthesized by BioWorld 2000 or the Mount Sinai Peptide Core Facility and were >85% pure as determined by ion-spray mass spectroscopy. The following peptides were produced, either linked to penetratin peptide (RQIKIWFQNRRMKWKK) (SEQ ID NO:32) or as the sequences shown: wt SSeCKS CY (LKKLFSSSGLKKLSGK) (SEQ ID NO:33), mutated CY (LSSSFSSSGLSSSSGK) (SEQ ID NO:34) or phosphoserine CY (LKKLFS^{Pi}SSGLKKLSPiGK) (SEQ ID NO:35). N-terminal biotinylation was performed on half the penetratin and half the non-penetratin-linked peptide product. Peptides were resuspended in DMEM and then incubated with cells at a final concentration of 100 µg/ml for 2-4 h. Peptide entry into cells was monitored by fixation of cells in ice cold ethanol/acetone (9:1) for 5 min at -20°C, washing with DMEM/10% CS, and incubation with PAb anti-cyclin D1, then with TRITC-labeled anti-rabbit Ig (Chemicon) and FITC-labeled avidin (Molecular Probes). Coverslips were mounted and photographed as described above.

Please **amend** the paragraph on p.113, line 6 to p.114, line 2 with the following paragraph:

Q7 SSeCKS binds G1 phase cyclins in vitro via tandem CY motifs. A so-called cyclin-binding (CY) motif which facilitates the binding of cyclins to several cell cycle components such as p21 (Chen et al., 1996, Mol. Cell. Biol. 16:4673-4682). SSeCKS encodes two closely spaced potential CY motifs, KKLFSxxxxKKLSG (SEQ ID NO:22) (K/RK/R followed by two nonpolar residues, with the first usually Leu). This domain also contains two major in vivo PKC sites, Ser507 and Ser515 (Lin et al., 1996, J. Biol. Chem. 271:28430-28438; Chapline et al., 1996, J. Biol. Chem. 271:6417-6422). It was tested whether a GST fusion protein containing the SSeCKS CY motifs ("SSeCKS-2") could bind G1 phase cyclins in an in vitro pulldown assay. Indeed, GST-SSeCKS-2, but not GST alone, bound endogenous and ectopic D1 from lysates prepared from S24, S24/D1, S24/V and V3 cells grown in the presence or absence of tet. Stripping of the blot and reprobing with cyclin E-specific antibody showed that GST-SSeCKS2 also bound cellular cyclin E. The levels of cyclins D1 or E bound by GST-SSeCKS2 corresponded to their relative stoichiometry in the cells tested, indicating saturation in the binding kinetics. Thus, higher amounts of D1 were bound in the S24/D1 cell lysates irrespective of tet conditions, whereas in S24 cells, where SSeCKS overexpression suppresses D1 levels, less D1 was bound in the [-]tet condition compared to the [+]tet condition. In contrast, the binding to cyclin E was relatively constant throughout the cells lines, reflecting the similar levels of cyclin E in these cells, whether in [-]tet or [+]tet conditions. Additionally, prephosphorylation of GST-SSeCKS2 with rabbit brain PKC (Upstate Biotechnology) ablated cyclin D and E binding.
